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The Effect of Aluminum on Cytokinins in the Mycelia of *Amanita muscaria*

Maja Kovač* and Jana Žel

National Institute of Biology, Karlovška 19, 61000 Ljubljana, Slovenia

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Abstract. High performance liquid chromatography analysis of immunoaffinity-purified extracts of mycelia of *Amanita muscaria,* and the *Amaranthus* bioassay of the eluted fractions, revealed the following seven cytokinins: zeatin, zeatin riboside, zeatin *N*-9-glucoside, dihydrozeatin, dihydrozeatin riboside, isopentenyl adenine, and isopentenyl adenosine. The decreased growth of aluminum-treated mycelia correlated with a 35% decrease in the total amount of the cytokinins. Among individual cytokinins, zeatin was the most affected, exhibiting a reduction of about 90%. The results are compared with previous investigations of aluminum effects on cytokinins in the mycelia of *Lactarius piperatus,* whose growth is stimulated by aluminum.

Aluminum toxicity can be a problem in acidic agricultural soils and also has been proposed as one of the main causes of forest decline (Ulrich et al. 1980). The physiology of Al effects on higher plants has been studied frequently (Taylor 1988, Luttge and Clarkson 1992), but there is very little evidence regarding the role of Al in mycorrhizal fungi (Thompson and Medve 1984).

There is some evidence that the effects of Al are mediated in part through effects on cytokinins (Pan et al. 1989, Čižkova, 1992). On the other hand, cytokinins added to the medium also influence the mycelial growth. The effect is dependent on the kind of hormone, the fungal species, and the concentration and medium used (Pokojska et al. 1993). Stimulation of fungal growth by cytokinins was observed more often than inhibition (reviewed in Gogala 1991; Pokojska et al. 1993).

The influence of Al on endogenous cytokinins has already been investigated in the mycelia of *Lactarius piperatus,* which is not only tolerant to very high concentrations of Al, up to 20 mM, but actually exhibits a stimulation of growth in response to Al (Žel and Gogala 1989, Kovač and Žel 1994). The aim of the present study was to determine the effect of Al on the endogenous cytokinins in the mycelia of *Amanita muscaria,* whose growth is inhibited by Al.

Materials and Methods

Mycelial Growth

Cultures of *L. piperatus* (L. ex. Fr.) Pers. ex. Hooker mycelia were grown on modified M-40 medium (5 g of malt extract, 5 g of glucose, 1.36 mg of KH_2PO_4, 0.5 g of NH_4Cl, 15 g of Difco Bacto Agar, and 1.2 ml of 1% $FeCl_3$ in 1 liter of double distilled water) supplemented with $Al_2(SO_4)3 \cdot 18H_20$ to give a final concentration of 0.1 mM Al. The same medium without Al was used as a control. The pH was adjusted to 4.5 before autoclaving. Cultures were grown in the dark at 25°C. For cytokinin analysis, mycelia were separated carefully from the media (using a spatula) after 21 days of growth.

Extraction and Purification

Ten to 30 g of fresh mycelia was ground with a prechilled mortar and pestle in 80% cold methanol. The cell debris was removed by filtering through Whatman No. 1 filer paper. Concentrated extracts were purified with polyvinylpolypyrrolidone at pH 3.1, followed by an immunoaffinity column prepared with polyclonal antibodies to zeatin riboside (ZR) and isopentenyl adenosine (iPA) (provided by B. Nicander, Swedish University of Agricultural Sciences) as described earlier (Dermastia and Kovač 1992, Kovač and Žel 1994). The antibodies used were capable of binding a wide range of cytokinins, including zeatin (Z), dihydrozeatin (DHZ), isopentenyl adenine (iP), and their corresponding

Abbreviations: ZR, zeatin riboside; iPA, isopentenyl adenosine; Z, zeatin; DHZ, dihydrozeatin; iP, isopentenyl adenine; DHZR, dihydrozeatin riboside; Z-9G, zeatin *N*-9-glucoside; iP-9G, isopentenyl *N*-9-glucoside; HPLC, high performance liquid chromatography; DHZRMP, dihydrozeatin riboside monophosphate; ZRMP, zeatin riboside monophosphate.

*Author for correspondence.

nucleosides, 9-glucosides, and nucleotides. The recoveries from the immunocolumns when a mixture containing 70 ng of each cytokinin was applied were as follows: Z, 100%; ZR, 100%; DHZ, 69%; dihydrozeatin riboside (DHZR), 67%; iP, 86%; iPA, 90%; zeatin *N*-9-glucoside (Z-9G), 100%; isopentenyl *N*-9-glucoside (iP-9G), 90%.

The affinity-purified materials were dried under vacuum redissolved in 500 µl of the starting mobile phase, and filtered before injection into high performance liquid chromatography (HPLC) columns.

HPLC Analysis

The cytokinins were fractionated on a 250- × 4-mm Supelco LC 18 DB column and monitored at 265 nm. A starting buffer of 0.1 M triethlammonium acetate containing a 10% mixture of methanol:acetonitrile (1:1, v/v) was used. The column was eluted at a flow rate of 1 ml/min using a gradient of 10–20% organic solvents over 25 min, 20% organic solvents to 30 min, and 20–30% to 40 min. The cytokinin activity of the eluted 1-ml fractions was detected by the *Amaranthus* betacyanin bioassay (Biddington and Thomas 1973).

An internal standard of H^3iPA was added to the extraction medium for recovery measurements, which on the average was 60–70%. No correction was made for losses.

The calculated cytokinin contents in the mycelia are the means of at least three independent experiments.

Results and Discussion

Figure 1 represents the high performance chromatogram of an immunoaffinity-purified extract of *A. muscaria* mycelia (Fig. 1A) and the results of an *Amaranthus* bioassay (Fig. 1B) of eluted fractions. The latter procedure is a very useful method for confirming the detection of biologic active cytokinins (Lough and Jameson 1992, Kovač and Žel 1994). HPLC analysis revealed the presence of at least seven cytokinins: Z-9G, Z, DHZ, ZR, DHZR, iP, and iPA. The presence of Z and ZR was confirmed by the *Amaranthus* bioassay. Very low biologic activity was found in the fractions corresponding to the retention times of DHZ, iP, and their ribosides. A lower response of these cytokinins in the *Amaranthus* bioassay compared with Z and ZR was also found in our previous experiments in which the betacyanin production of the standard cytokinins was estimated (Kovač and Žel 1994). The peak eluted from HPLC at the retention time of the biologic inactive cytokinin Z-9G was confirmed further by comparing its UV spectrum with the UV spectrum of the standard. The peak eluted at 10.54 min, which is the retention time of dihydrozeatin riboside monophosphate (DHZRMP) and zeatin riboside monophosphate (ZRMP) was not a cytokinin nucleotide, as it was inactive in the *Amaranthus* bioassay. Their rather high biologic activity was demonstrated in our previous experiments

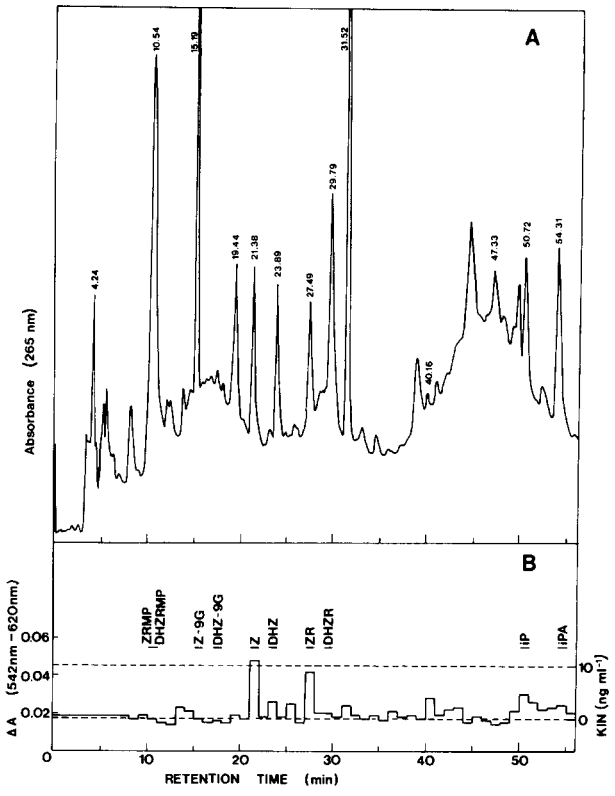

Fig. 1. HPLC of immunoaffinity-purified extract of 0.7 g, dry weight, of *A. muscaria* mycelia (**A**) and the *Amaranthus* bioassay of 1-ml fractions (**B**). Retention times of the standards are given by bars. Dotted lines indicate the control (0) and kinetin (KIN) on betacyanin production.

(Kovač and Žel 1994). The other unidentified peaks shown in Figure 1A might be cytokinins that we were unable to identify or other substances that interfered with the antibodies used. In comparison with the cytokinins present in the mycelia of the mycorrhizal fungus *L. piperatus* (Kovač and Žel 1994), more cytokinins were detected in the mycelia of *A. muscaria* as Z-9G, DHZR, and iPA were not found in the former species.

Aluminum present in the medium inhibited lateral growth and fresh and dry weights of the mycelia of *A. muscaria*, as was also seen in our previous paper (Žel et al. 1992). The influence of 0.1 mM Al on the endogenous cytokinins of *A. muscaria* mycelia estimated from the HPLC peak area and the bioassay is presented in Table 1. Although the *Amaranthus* bioassay cannot be used for precise cytokinin quantification because of different sensitivities to various cytokinins, a reduction of the total amount of cytokinins in treated mycelia was found using both methods. The total amount of cytokinins calculated by the integration of HPLC peaks was reduced by 35% in treated mycelia. Among individual cytoki-

Table 1. Effect of 0.1 mM Al on endogenous cytokinins of *A. muscaria* mycelia calculated from the HPLC peak area (ng/g, dry weight) and from the *Amaranthus* bioassay (ng of kinetin equivalents/g, dry weight).

	Control[a]		0.1 mM Al[a]	
Cytokinin	ng/g DW[b]	ng KIN eq./g DW[c]	ng/g DW	ng KIN eq./g DW
Z	25.7 ± 3.8	23.7 ± 1.8	1.7 ± 0.9	9.8 ± 1.5
ZR	20.5 ± 8.9	30.2 ± 9.4	10.0 ± 4.6	13.8 ± 4.0
Z-9G	42.9 ± 9.6	ND[d]	28.5 ± 6.6	ND
DHZ	10.5 ± 6.1	ND	4.4 ± 2.7	2.9 ± 0.8
DHZR	59.5 ± 12.5	ND	43.1 ± 9.6	2.9 ± 1.4
iP	9.7 ± 4.3	18.2 ± 3.4	1.2 ± 0.7	ND
iPA	59.0 ± 13.5	20.4 ± 0.3	56.4 ± 19.6	5.3 ± 1.6
Total	227.8	92.5	145.3	34.7

[a] Each value represents the mean of at least three independent experiments (mean ± S.E.).
[b] DW, dry weight.
[c] KIN eq., kinetin equivalents.
[d] ND, not detectable.

nins, Z was the most affected by Al, as about an 90% reduction was observed. In these experiments, higher concentrations of Al (1 or 10 mM) inhibited the growth of mycelia so much that there was not enough material for analysis.

In our previous experiments the opposite effect of Al on some physiologic processes of *L. piperatus* and *A. muscaria* mycelia was shown. Al incorporated in the medium stimulated the growth of *L. piperatus*, whereas the growth of *A. muscaria* was inhibited (Žel and Gogala 1989, Žel et al. 1992). Al also induced a relative enlargement of the less ordered domains in the membranes of *L. piperatus* mycelia (Žel et al. 1993a) and decreased that of *A. muscaria* (Žel et al. 1993b). The effect of Al on the content of Ca, P, Mg, and K was similar in both mycelia, but the acceptance of Al from the media was higher in *L. piperatus* than in *A. muscaria* (Žel and Bevc 1993). The difference between the two fungi was also reflected in the studies of the Al effect on cytokinins in mycelia where 10 mM Al increased the total amount of cytokinins in *L. piperatus* (Kovač and Žel 1994) in contrast to the findings of this study, where 0.1 mM Al already decreased its amount in *A. muscaria*. The same effect was found in individual cytokinins. One explanation for the difference in the reaction of the two fungi might be that the mycelia of *L. piperatus* grown on the control media contain twice as much Ca and three times less P (Žel and Bevc 1993). It was demonstrated that calcium ions lessen Al toxicity and that the ratio of Al to Ca probably determines the extent of deleterious Al effects (reviewed in Rengel 1992a).

There is much evidence that both Al and cytokinins affect mitosis, the former as an inhibitor, the second as a promoter. Both effects seem to be mediated through Ca^{2+} as a second messenger (reviewed in Rengel 1992a, 1992b and Saunders 1990). Based on the above findings, although they are mostly from higher plants, we suppose that in the case of *A. muscaria*, both Al and the decrease of cytokinins lead to reduced growth, probably by altering the cytosolic Ca. The reduction of cytokinins might be the consequence of Al impairment of cytokinin biosynthesis or the result of the reduced growth of the stressed fungi regulated by another mechanism.

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